EXPERIMENTAL EVALUATION OF THE PULMONARY EDEMA INDUCED BY THE VENOM OF THE SCORPION *TITYUS ASTHENES* IN RATS

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Abstract

The species *Tityus asthenes* has been responsible for scorpion sting deaths in Panama. Pulmonary edema is one of the main causes of death registered by scorpionism. In the present work, we determined the capacity of the venom of the scorpion *Tityus asthenes* to induce pulmonary edema in rats. The ability of *T. asthenes* venom to induce acute pulmonary edema in rats was determined using four approaches: (1) the difference in wet weight using the lung index between treated and untreated lungs; (2) histological analysis; (3) changes in pulmonary vascular permeability; and (4) total leukocyte count obtained from bronchoalveolar lavage. We found that histological sections of venom-treated lungs showed moderate pulmonary edema, and an increase in total leukocyte count compared to control samples. However, the pulmonary index and the vascular permeability of venom-treated lungs were similar to those of control samples. We conclude that the venom of *T. asthenes* scorpions can induce moderate pulmonary edema in rats. The experimental model was validated for future studies on the pathophysiology of pulmonary edema caused by the venom of scorpions of the genus *Tityus* in Panama.
Keywords: Bronchoalveolar lavage, Evans blue, leucocytes, phenylbiguanide, pulmonary index.

INTRODUCTION

In the last decades, scorpionism in Panama has had a significant increase, from less than 400 cases in 2000 to more than 4000 cases in 2016 and has now become a public health problem (Arjona, 2017). The scorpions involved in deaths from stings in Panama belong to the genus *Tityus* (Lourenço and Méndez, 1984; Borges et al, 2011). The species *Tityus asthenes* has been involved in human deaths from scorpion stings in the Republic of Panama (Borges et al, 2011; Arjona, 2017; Ministerio de Salud de Panama, 2018). The envenomation caused by Tityus scorpions induces damage in almost all organs. However, lethal damage occurs in the lungs mainly due to acute pulmonary edema (APE), which is one of the main causes of death (Rahav and Weiss, 1990; Amaral et al, 1994; Ismail, 1994; de Dàvila et al, 2002; Jiménez and Rosales, 2003; Razi and Malekanrad, 2008).

The pathophysiology of lung damage is still controversial and needs to be further investigated. Various authors maintain that the nature of pulmonary edema has a cardiogenic origin, attributed to acute left ventricular failure (Gueron et al, 1990; Amaral et al, 1993; Bucaretchi et al, 1995; Gueron and Ilia, 1996; de Dàvila et al, 2002).Other researchers propose that the edema is of non-cardiogenic origin, which is due to an increase in the permeability of the pulmonary alveolocapillary membrane or acute respiratory distress syndrome (ARDS), accompanied by an increase in IL-1α , IL-6, IL-10α, TNF-α and IL1-β (Rahav and Weiss, 1990; Müller, 1992; Amaral et al, 1993; Freire-Maia and de Matos, 1993; Gueron and Ilia, 1996).The current study sought to determine the ability of *T. asthenes* venom to cause acute pulmonary edema in rats.

MATERIALS AND METHODS

Animals: Sprague Dawley rats (6-8 weeks) of both sexes (180-250 g) were obtained from the Bioterium of the Institute of Scientific Research and High Technology Services of Panama (INDICASAT, Panama City, Panama). The animals were kept at a temperature of 22 ºC, food and water were supplied *ad libitum*. All the tests carried out were approved (Note N° CEIBAUP-019-2020) by the Research Ethics and Animal Welfare Committee (CEIBA) at the University of Panama, (Panama City, Panama).
Scorpion venom and drugs: *T. asthenes venom* (lyophilized) was provided by the Center for Research and Information on Medicines and Toxics (CIIMET, Panama City, Panama). The following drugs and reagents were obtained from their respective manufacturers: phenylbiguanide (ChemScene, NJ, USA), bovine serum albumin (Sigma Aldrich, MO, USA), atropine (PiSA, CDMX, MEX), ketamine (Vets Pharma, CDMX, MEX), xylazine hydrochloride (Biomont, LIM, PE), and sodium pentobarbital (Biomont (INVET, BTA, COL).

Experimental protocol: To determine the potential of *T. asthenes* venom to induce acute pulmonary edema in rats, we followed the following techniques.

A. **Evaluation of pulmonary edema induction**

Eighteen rats were divided into three experimental groups: group # 1 (N = 6): (1 mg/kg venom) + atropine (1 mg/kg) to prevent apnea and bradycardia, caused by the venom (Freire-Maia et al, 1973,1974); group #2 negative control Phenylbiguanide (PBG) with two subgroups (N=3): (10 µg/kg) + atropine (1 mg/kg), (N = 3): (50 µg/kg) + atropine (1 mg/kg) and group #3 control (N=6): 200 mL of 0.9% saline.

The rats were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine (75 mg/kg) intraperitoneally (i.p.). All substances were administered intravenously (i.v.) through a cannula inserted into the femoral vein. The animals were observed one hour after treatment and immediately sacrificed with a dose of sodium pentobarbital (10 mg/kg). The lungs were extracted and weighed, in an analytical balance (Mettler Toledo, OH, USA) and the pulmonary index was determined using the formula: pulmonary index = weight of the lungs x100 / body weight (Magalhães et al, 1998).

B. Histology: The lungs of the animals were fixed with 10% formalin. They were cut to a thickness of 4 mm. The sections were stained with hematoxylin eosin (HE); mounted on slides and viewed at 40x in an optical microscope (LEICA, DM500). After the morphological analysis, the tissues were classified into normal tissue and tissue with the presence of APE, according to De Matos et al, (1997) and modified by Oliveira et al, (2013).

C. Vascular permeability changes: To measure changes in pulmonary vascular permeability, the Evans blue protocol was used (Saria and Lundberg, 1983; De-Matos et al, 2001). Before venom, drug, and saline administration, Evans blue (20 mg/kg) was administered. After one hour, the animals were sacrificed with an overdose of sodium pentobarbital (10 mg/kg) and an endotracheal cannula was placed for bronchoalveolar lavage (BAL) with a 5 mL saline solution (Henderson, 2005; Van Hoecke et al., 2017). The BAL was collected and centrifuged (Eppendorf, 5804R) at 1000 rpm for 7 minutes. The supernatant was used to determine the concentration of Evans blue by spectrophotometry at 620 nm (BioTek, Epoch) and the pellet was preserved for the leukocyte count.

D. Total leucocyte count: The BAL pellet from the animals was resuspended in 1 mL of phosphate buffered saline (PBS) (0.1 M) containing 3% bovine serum albumin and an aliquot (20 µL) diluted in Türk solution Oliveira et al, (2013), where the BAL pellet from the three groups was resuspended in 1 mL of phosphate buffered saline (PBS) (0.1 M) containing 3 % bovine serum albumin and an aliquot (20 µL) diluted in Türk solution. The total leukocyte count was performed in a Neubauer chamber using an optical microscope (LEICA, DM500) under a 40x objective. The total number of leukocytes/mm3 was determined by the ratio A/DV (where A is the total leukocyte count in the four quadrants, D is the dilution used, and V is the volume count used). D and V are constant.

Statistical analysis: Pulmonary index, Evans blue, and leukocyte results were analyzed using the Mann–Whitney U test. These data are shown as mean ± SEM with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

A. Induction of pulmonary edema: The lungs of rats injected with *T. asthenes* scorpion venom (1 mg/kg, i.v.) did not show an increase in weight when compared with the control group and with the PBG 10 mg/kg negative group ($p > 0.47$; Table1).

TABLE 1. Pulmonary index of the experimental groups: control (200 µL saline solution), PBG (10 µg/kg), PBG (50 µg/kg) and *T. asthenes* venom (1 mg/kg). Presented as mean and SEM.

GROUPS (N)	PULMONARY INDEX (□ ± SEM)
Control (6)	1.08 ± 0.08
Venom (1mg/kg) (6)	1.01 ± 0.05
PBG □□□*µ*/kg (3)	1.03 ± 0.03
PBG 50 *µ*g/kg (6)	1.23 ± 0.09*

* Significant difference

On the other hand, the PBG 50 mg/kg group presented an increase in weight change when compared with the control group and with the group treated with *T. asthenes* venom ($p < 0.04$).

B. Histology: Histological examination revealed that the lung tissues of the venom-treated rats had moderate pulmonary edema with multifocal alveolar spaces, neutrophilic vasculitis, emphysema, lymphoid hyperplasia, and alveolar erythrocytes. The PBG group had mild pulmonary edema, moderate emphysema, suppurative vasculitis, and alveolar fibrin. The control group, on the other hand, had normal tissue with perivascular and intraalveolar hemorrhages, which were most likely caused by prolonged manipulation of the lungs during weighing (Fig. 1).

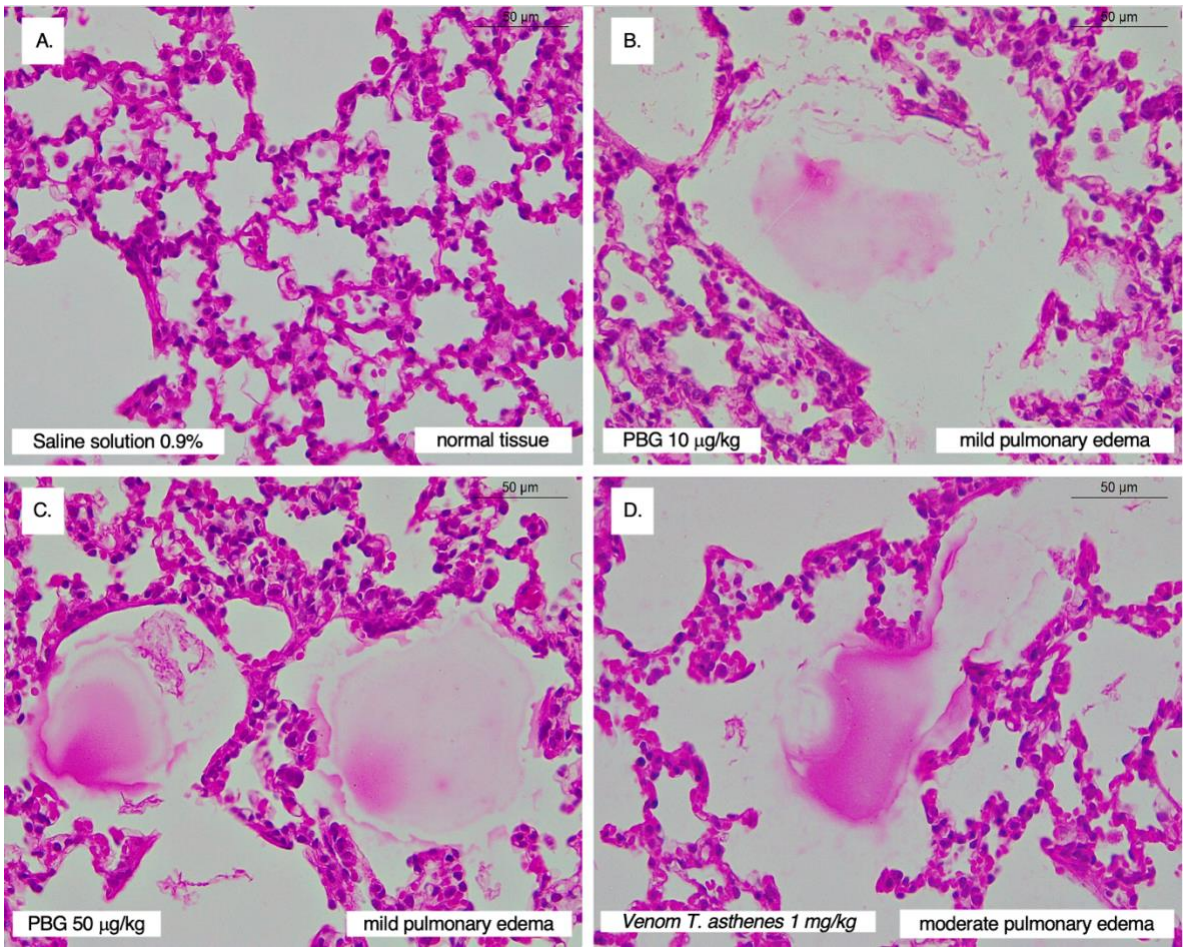


Figure 1**.** Histological sections (40X) of rat lungs one hour after i.v. injection with (**A**), 0.9% saline solution (200 µL), (**B**) PBG (10 µg/kg), (**C**), PBG (50 µg/kg), and (**D**) *T. asthenes* venom (1 mg/kg), using an optical microscope under a 40x objective.

C. Measurement of vascular permeability changes: Pulmonary vascular permeability was not significantly increased in animals treated with scorpion venom and PBG at 10 mg/kg and 50 mg/kg (Fig. 2) when compared to the saline group ($p > 0.05$). However, we have documented that those animals injected with *T. asthenes* venom showed a clearance in Evans blue fluid extravasation.

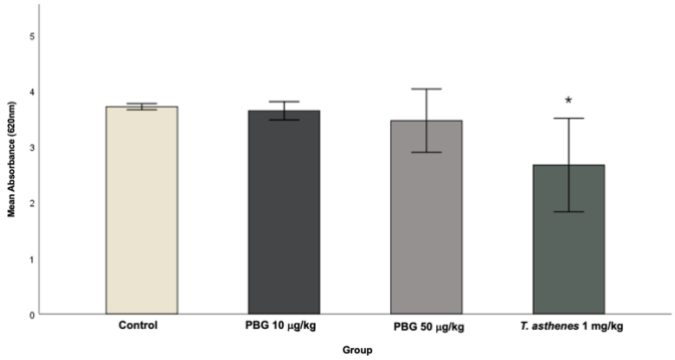
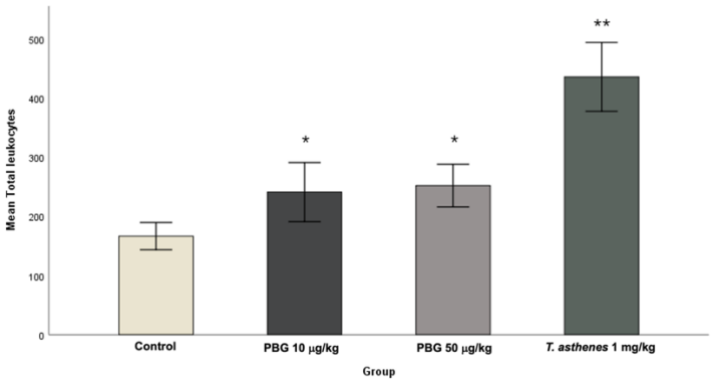


Figure 2. Pulmonary vascular permeability measured as mean absorbance (mean ± SEM), of **(A),** the control group 200 □L saline 0.9%), and experimental groups: **(B)**, PBG (10 □g/kg), **(C)**, PBG (50 □g/kg) and the **(D),** *T. asthenes* venom-injected group (1 mg/kg).

D. Total leukocyte count: animals treated with *T. asthenes* venom showed an increase in the total leukocyte count present in the bronchoalveolar lavage of (Fig. 3), compared to the control group and with the PBG-treated group ($p < 0.004$). Similarly, when compared to the control group, animals treated with PBG at 10 mg/kg and 50 mg/kg showed differences ($p < 0.019$).



*p<0.019, **p<0.004

Figure 3. Total leukocyte count of the experimental groups, **(A),** control group (200 µL saline 0.9%), **(B),** PBG (10 µg/kg), **(C),** PBG (50 µg/kg) and the **(D),** *T. asthenes* venom (1 mg / kg). Presented as mean and SEM.

In this first study on the pathophysiology of *T. asthenes* venom, we found that pulmonary edema is induced one hour after intravenous injection of the venom (1 mg/kg). The most important components of Tityus scorpions venoms for the intoxication effects in humans, are the neurotoxic peptides of the type □ □and □. These peptides act on the Na_v ion channels in subunits 3 and 4, which alters the inactivation mechanism, increasing the release of neurotransmitters such as acetylcholine, norepinephrine and epinephrine, which in turn, affect both the sympathetic and parasympathetic nervous systems (Couraud et al, 1982; Becerril et al, 1997; Cestele et al, 1998; Pintar et al, 1999; Possani et al, 1999; Batista et al, 2007). The pulmonary index obtained from rats treated with the *T. asthenes* venom is consistent with the results of Freire-Maia and de Matos, (1993) using the venom of *Tityus serrulatus*. Also, the histology of the lung tissues revealed the presence of moderate pulmonary edema in animals treated with the *T. asthenes* venom, as described in previous studies using the venom of the scorpions *T. serrulatus*, *Buthus tamulus*, and *Androctonus australis hector* at different doses (De Matos et al, 1997; Deshpande et al, 1999; Adi-Bessalem et al, 2012; Oliveira et al, 2013; Chaïr-Yousfi, et al, 2015; Medjadba et al, 2016). The increase in the mean lung index of the negative control group could be associated with methodological errors before weighing.

As expected, pulmonary edema was documented for animals treated with PBG at doses of 10 and 50 µg/kg. In the study carried out by Dutta and Deshpande (2010) an increase in the water content in the lungs is described using a dose of 10 µg/kg. In addition, they showed that when using PBG in the range of 10 µg/kg - 100 µg/kg similar cardiorespiratory values are obtained (Dutta et al, 2012).

Pulmonary edema caused by the effects of scorpion venom in the body has a cardiogenic component. There is an increase in circulating catecholamines with a stimulus in the adrenal glands and sympathetic nerve terminals, with acute left ventricular failure being the main cause of pulmonary edema acute (Amaral et al, 1993; Bucaretchi et al, 1995; de Dàvila et al, 2002). The non-cardiogenic origin of pulmonary edema is associated with vasoactive substances such as bradykinin, prostaglandin, histamine, and the activation of neuropeptides such as substance P, which act in the release of inflammatory mediators, increasing pulmonary vascular permeability (De Matos et al, 1997; Oliveira et al, 2013).

An increase in pulmonary vascular permeability was not observed in rats injected with *T. asthenes* venom, failing to support a non-cardiogenic origin of the edema. The different values obtained from Evans blue extravasation in bronchoalveolar lavage could be explained by an inhibition of the platelet activating factor since it is believed to be involved in the formation of pulmonary edema. Another possibility in the genesis of pulmonary edema would be the inhibition of the □ subunit of the alveolar epithelial Na^+ channel and aquaporin 5 and a greater activity of the Na-K-2Cl cotransporter, which could cause the clearance of pulmonary fluid (Malaque et al, 2015; Miyamoto et al, 2018).

Our results showed an increase in leukocytes (leukocytosis) obtained from bronchoalveolar lavage. This leukocytosis is observed in human poisonings due to the scorpion sting (Coronado et al., 2008). The increase in leukocytes may be associated with the activation of the inflammatory cascade activated by the toxins *T. asthenes* (Adi-Bessalem et al, 2012; Chaïr-Yousfi et al, 2015; Oliveira et al, 2013). However, more detailed studies are needed to find conclusive answers about the effect of this venom on the development of pulmonary edema.

Conclusion

In conclusion, the administration of the *T. asthenes* venom in rats caused moderate pulmonary edema. These preliminary findings confirm the validity of the experimental protocol for studying the pathophysiology of *Tityus* scorpion venom at the pulmonary level in our country, which will help us develop future research.

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